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Low doses of paraquat and polyphenols prolong life span and locomotor activity in knock-down *parkin Drosophila melanogaster* exposed to oxidative stress stimuli: Implication in autosomal recessive juvenile Parkinsonism

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ABSTRACT

Previous studies have shown that polyphenols might be potent neuroprotective agents in *Drosophila melanogaster* wild type Canton-S acutely or chronically treated with paraquat (PQ), a selective toxin for elimination of dopaminergic (DAergic) neurons by oxidative stress (OS), as model of Parkinson's disease (PD). This study reports for the first time that knock-down (K-D) *parkin Drosophila melanogaster* (TH-GAL4; UAS-RNAi-*parkin*) chronically exposed to PQ (0.1–0.25 mM), $FeSO_4$ (Fe, 0.1 mM), deferoxamine (DFO, 0.01 mM) alone or (0.1 mM) PQ in combination with polyphenols propyl gallate (PG, 0.1 mM) and epigallocathecin gallate (EGCG, 0.1, 0.5 mM) showed significantly higher life span and locomotor activity than untreated K-D flies or treated with (1, 5, 20 mM) PQ alone. Whilst gallic acid (GA, 0.1, 0.5 mM) alone or in the presence of PQ provoked no effect on K-D flies, epicathecin (EC, 0.5 mM) only showed a positive effect on prolonging K-D flies' life span. It is shown that PG (and EGCG) protected protocerebral posterolateral 1 (PPL1) DAergic neurons against PQ. Interestingly, the protective effect of low PQ concentrations, DFO and iron might be explained by a phenomenon known as "hormesis." However, pre-fed K-D flies with (0.1 mM) PQ for 7 days and then exposed to (0.25 mM) for additional 8 days affect neither survival nor climbing of K-D *Drosophila* compared to flies treated with (0.25 mM) PQ alone. Remarkably, K-D flies treated with 0.1 mM PQ (7 days) and then with (0.25 mM) PQ plus PG (8 days) behaved almost as flies treated with (0.25 mM) PQ. Taken these data suggest that antioxidant supplements that synergistically act with low pro-oxidant stimuli to prolong and increase locomotor activity become inefficient once a threshold of OS has been reached in K-D flies. Our present findings support the notion that genetically altered *Drosophila melanogaster* as suitable model to study genetic and environmental factors as causal and/or modulators in the development of autosomal recessive juvenile Parkinsonism (AR-JD)/PD. Most importantly, we have shown for the first time that low amounts of stressors induce a health-promoting extending effect in K-D *parkin* flies. Altogether our present results open new avenues for the screening, testing and development of novel antioxidant drugs against OS stimuli in neurodegenerative disorders.

1. Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder clinically characterized by bradykinesia, rigidity, resting tremor, and postural instability (Jankovic, 2008). These motors defects arise as a consequence of the loss of 50–70% of dopaminergic neurons located in the *substantia nigra*, decrease of the neurotransmitter dopamine content in striatum, cytoplasmic inclusions of insoluble, aggregated proteins known as Lewy bodies, and elevated levels of iron (Cuervo et al., 2010; Forno, 1996; Sian-Hülsmann et al., 2011). PD is a multifactorial disorder probably arising from polygenic inheritance, exposure to environmental toxins, and gene–environmental interactions. Despite these assumptions, >90% of PD cases are of sporadic nature, and ~5–10% are caused by gene mutations given rise to rare familial forms of the disease. Until now, mutations in six genes have conclusively been demonstrated to cause either autosomal recessive juvenile Parkinsonism (AR-JP), i.e., *PARKIN*, *DJ-1*, phosphatase and tension homolog (PTEN)-induced kinase 1 (*PINK1*), P-type ATPase (*ATP13A2*), or autosomal dominant Parkinsonism, i.e., *α-SYNUCLEIN* (non-A4 component of amyloid precursor (*SNAC*) and leucine repeat rich kinase 2 (*LRRK2*) (Bekris et al., 2010; Nuytemans et al., 2010). Interestingly, *PARKIN* mutations account for >50% of patients with AR-JP and reported pathogenic mutations (>200; http://www.molgen.vib-ua.be/PDMutDB) include breakpoints, missense and nonsense mutations, deletions, rearrangements and duplications. The *PARKIN* gene is located on chromosome 6 (6q25.2-q27), contains 12 exons and encodes a 465-amino acid protein known as Parkin. The Parkin belongs to the E3 ubiquitin ligase subset of

Abbreviations: AR-JP, autosomal recessive familial Parkinsonism; DFO, deferoxamine; DAergic, dopaminergic; EC, epicathecin; EGCG, epigallocathecin gallate; GA, gallic acid; GFP, green fluorescent protein; OS, oxidative stress; PQ, Paraquat; PD, Parkinson's disease; PG, propyl gallate; ROS, reactive oxygen species.

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the RBR (RING, in between RING, RING) protein family involved in, but not restricted to, protease degradation (Rankin et al., 2011). Unfortunately, treatment is neither available for sporadic nor for genetic forms of Parkinsonism. The therapy consists only of amelioration of the symptoms by replacement of deficient dopamine (Jankovic and Aguilar, 2008). Therefore, other treatment approaches are critically needed for AR-JP/PD patients.

Because bioethical limitations of biologic and genetic analysis in humans, most of these studies have been performed in model organism such as the fly *Drosophila melanogaster* (Muñoz-Soriano and Paricio,

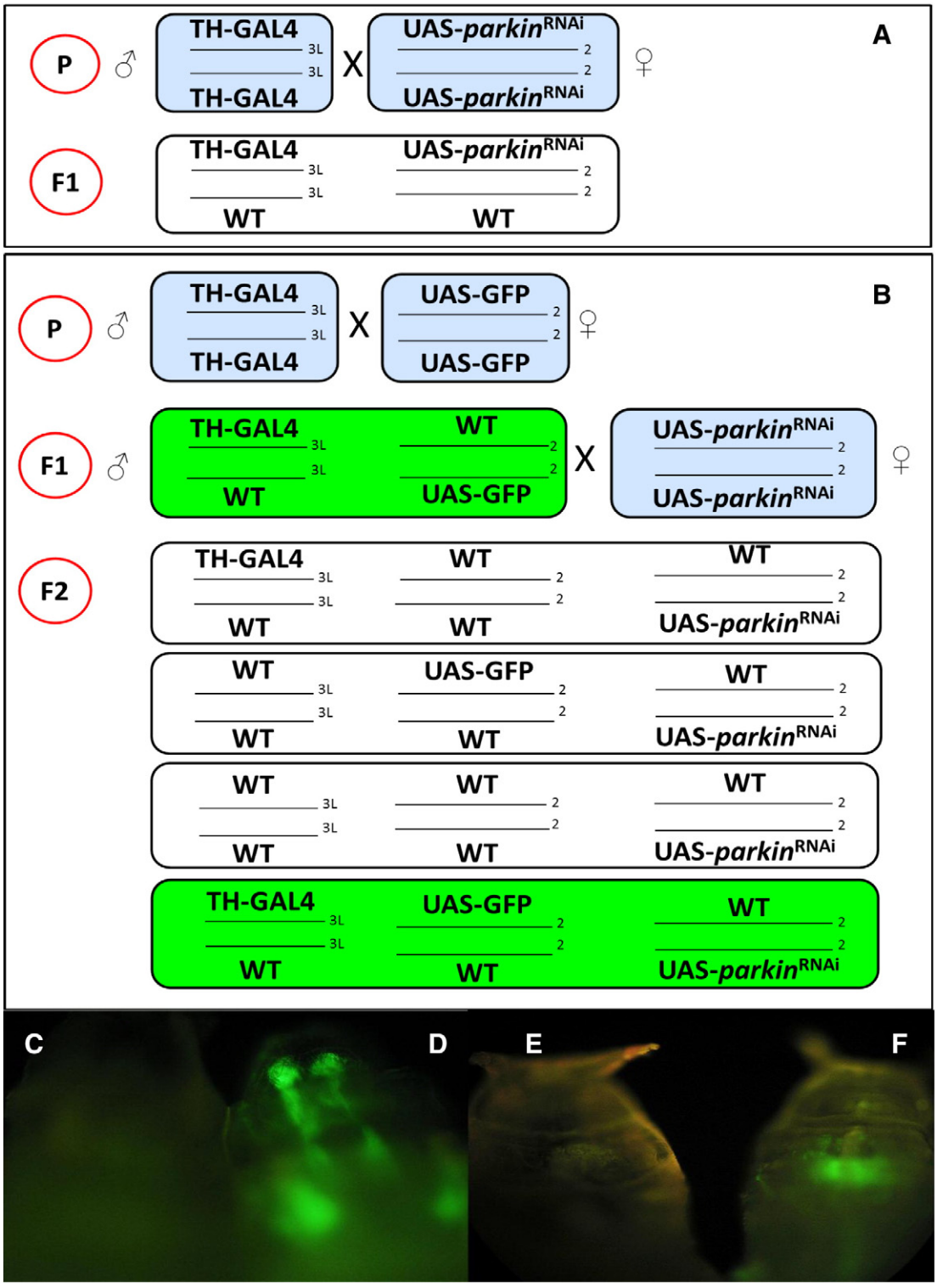

Fig. 1. Scheme for basic fly cross and selection. (A) TH-GAL4/UAS-RNAi-*parkin* flies were obtained by crossing TH-GAL4 (male, $n = 10$) and UAS-RNAi-*parkin* (female, $n = 10$). After 5 days of husbandry, parental flies are retired and discarded form mating tubes. F1 was then reared according to standard procedures. (B) Green fluorescent protein (GFP) positive TH-GAL4/UAS-RNAi-*parkin* (female) flies were obtained by double crosses. Parental TH-GAL4 (male) X UAS-GFP results in F1 fluorescent individuals. Adults F1 (male) were crossed with UAS-RNAi-*parkin* (female) to obtain an F2 comprising one out of four GFP^{+}/GAL4/RNAi-*parkin* individuals from the fly population. (C–E) Photography shows a negative $GFP^{(-)}$/GAL4/RNAi-*parkin* and (D and F) positive $GFP^{(+)}$/GAL4 RNAi-*parkin* larvae and pupae, respectively, for illustrative process of fly selection.

2011). Indeed, several studies have shown that specific genetic alteration (Clark et al., 2006; Feany and Bender, 2000; Lavara-Culebras and Paricio, 2007; Lee et al., 2007; Pendleton et al., 2002) and pharmacological treatment (Chaudhuri et al., 2007; Coulom and Birman, 2004) in *Drosophila* could be used to model sporadic and familial PD. Specifically, systemic *parkin* null mutants or overexpression of mutated *parkin* in dopaminergic neurons result in mitochondrial defects, hypersensitivity to oxidative stress, reduced life span, degeneration of dopaminergic neurons, loss of locomotor abilities (Greene et al., 2003; Pesah et al., 2004; Sang et al., 2007; Wang et al., 2007). Despite these advances, no pharmacological treatments have been attempted in *D. melanogaster* flies with null *parkin* in dopaminergic nuclei, as a valid approach to the treatment of AR-JP patients.

Paraquat (PQ) is a pesticide which specifically destroys dopaminergic neurons in mammalian (Kuter et al., 2007; Li et al., 2005) and non-mammalian (Bretaud et al., 2004; Chaudhuri et al., 2007) organisms, most probably through interaction with mitochondrial complex I (Cocheme and Murphy, 2008; Sanz et al., 2010), generation of reactive oxygen species (ROS), oxidative stress (OS) and cell death (Bonilla et al., 2006; Dinis-Oliveira et al., 2006). Due to the specificity with which PQ targets the nigrostriatal DAergic system (Thiruchelvam et al., 2000), it has become a popular neurotoxin to induce Parkinsonism and pre-clinical assays in PQ-intoxicated *D. melanogaster*. Indeed, Peng and co-workers have shown that chronic PQ exposure shortened the maximum survival time from 73–61 to 35–31 days and decreased the climbing ability by 60%, while blueberry extracts at 5 mg/ml (Peng et al., 2012) or apple polyphenols (Peng et al., 2011) in diet could significantly increase the survival rate and partially restore the climbing ability of Oregon-R wild type *Drosophila* flies. In accordance, we have shown that pure polyphenols prolonged life span, rescue and restore locomotor activity impairment in Canton-S wild type *D. melanogaster* from acutely or chronically exposed to PQ (Jimenez-Del-Rio et al., 2010; Ortega-Arellano et al., 2011). We also showed that high concentrations of iron were able to diminish fly survival and locomotor activity over a period of 5 days (Jimenez-Del-Rio et al., 2010). Remarkably, polyphenols protected and maintained movement abilities in flies co-treated with PQ and iron (Jimenez-Del-Rio et al., 2010). Taken together these data suggest *Drosophila* fly as suitable model to study antioxidant drugs or compounds against OS stimuli in neurodegenerative disorders (Surendran and Rajasankar, 2010). However, until now no data is available to establish whether polyphenols might have potential in the treatment of AR-JP, especially due to *parkin* gene alterations. Based on the aforementioned reports, we hypothesized that polyphenols can positively affect life span and locomotor activity on knock-down (K-D) *parkin* flies chronically treated with PQ.

To test this assumption, we first created a filial 1 (F1) K-D *parkin D. melanogaster*, as illustrated in Fig. 1A. The aims of the present work were (1) to study the life span and locomotor activity (i.e. climbing capability) of F1 K-D *parkin* flies chronically exposed to increasing concentrations of PQ alone upon 1% glucose feeding regimen for 15 days;

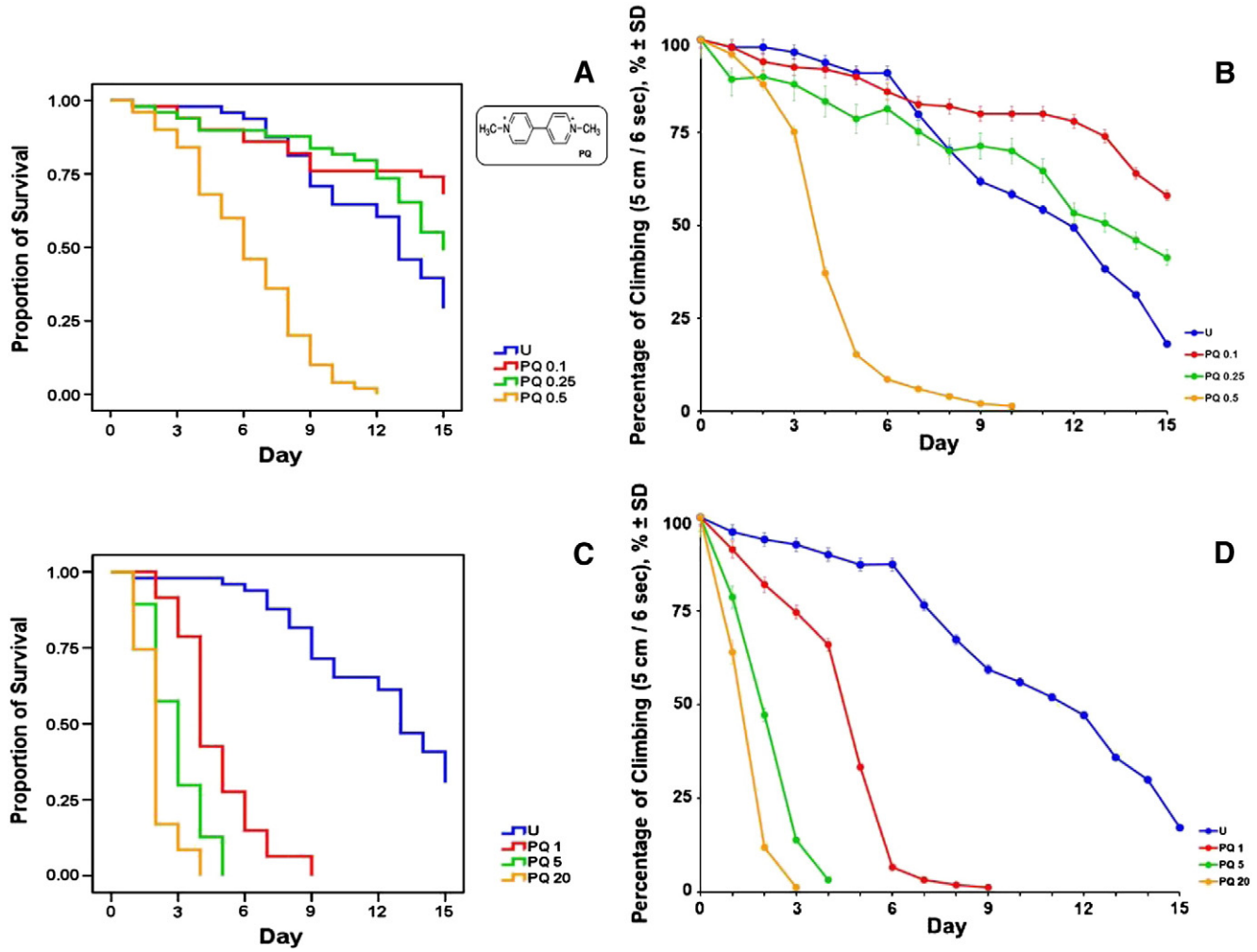

Fig. 2. Effect of low and high concentrations of paraquat on the survival (A and C) and locomotor activity (B and D) of knock-down (K-D) *parkin Drosophila melanogaster* in the absence (0, blue bar) or presence of paraquat (PQ: 0.1, 1 (red), 0.25, 5 (green), 0.5, 20 mM (orange bar)). Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A and C) $P<0.05$ by log-rank test and (B and D) $P<0.05$ by χ^2 test.

(2) to determine whether polyphenols such as propyl gallate (PG), gallic acid (GA), epicatechin (EC), and epigallocatechin-3-gallate (EGCG), iron (i.e., $FeSO_4$) and deferoxamine (DFO, an iron chelator) affect the life span and locomotor activity of the genetically altered F1 flies exposed to PQ for the same period of time. Further, we investigate whether PG was able to protect dopaminergic (DAergic) neurons against PQ in K-D flies (Fig. 1B). Based on our present findings, it is proposed that some polyphenol and chelating agents should provide to pre-clinical genetically individuals at risk to suffer AR-JP a means to delay or to prevent motor symptoms and/or frank PD, as reported in Antioquia, Colombia (Pineda-Trujillo et al., 2001, 2006, 2009). These data may contribute to a better understanding of the inherent genetic predisposition and environmental agents as causative factors of PD, and its potential treatment.

2. Materials and methods

2.1. Fly stock and culture

TH-GAL4 (Bloomington Stock Center #8848), and UAS-RNAi-*parkin* (Vienna Drosophila RNAi Center #47636) *Drosophila melanogaster* flies were maintained at 25 °C on 12 h light/dark cycle in bottles containing agar, corn meal, molasses, water, and dried yeast medium. Propionic acid was added to prevent fungal growth (Merck Schuchardt OHG, D-85662 Hohenbrunn, Germany) and other reagents unless specified otherwise were purchased from Sigma (St. Louis, MO, USA). Knock-down flies (e.g. F1) were obtained by crossing male (BSC#8848) and female (VDRC#47636; Fig. 1A). Knock-down (K-D) female (f) flies F1 (fF1) were collected for experimentation under brief CO_2 anesthesia from 2 to 3 days after eclosion.

2.2. Paraquat toxicity assay

The paraquat toxicity assay was performed on virgin 2- to 3-day-old flies collected overnight and kept on regular food medium. Subsequently, 50 separated adult fF1 flies were starved in empty vials for 3 h at 25 °C. Then, groups of five flies were placed in 10 vials containing a filter paper (Bio Rad Mini Trans-Blot 1703932) saturated with 1% glucose (GLU, 55.5 mM) in distilled water (dW) for 24 h. After this time, flies were starved in empty vials for 3 h at 25 °C and transferred to vials with a filter paper saturated with 200 µl (increasing concentrations) PQ in 1% GLU for 15 days. Red food dye (8 µl/1 ml) (Red food color McCormick) was added to ensure homogeneity and food intake. Living flies were counted daily.

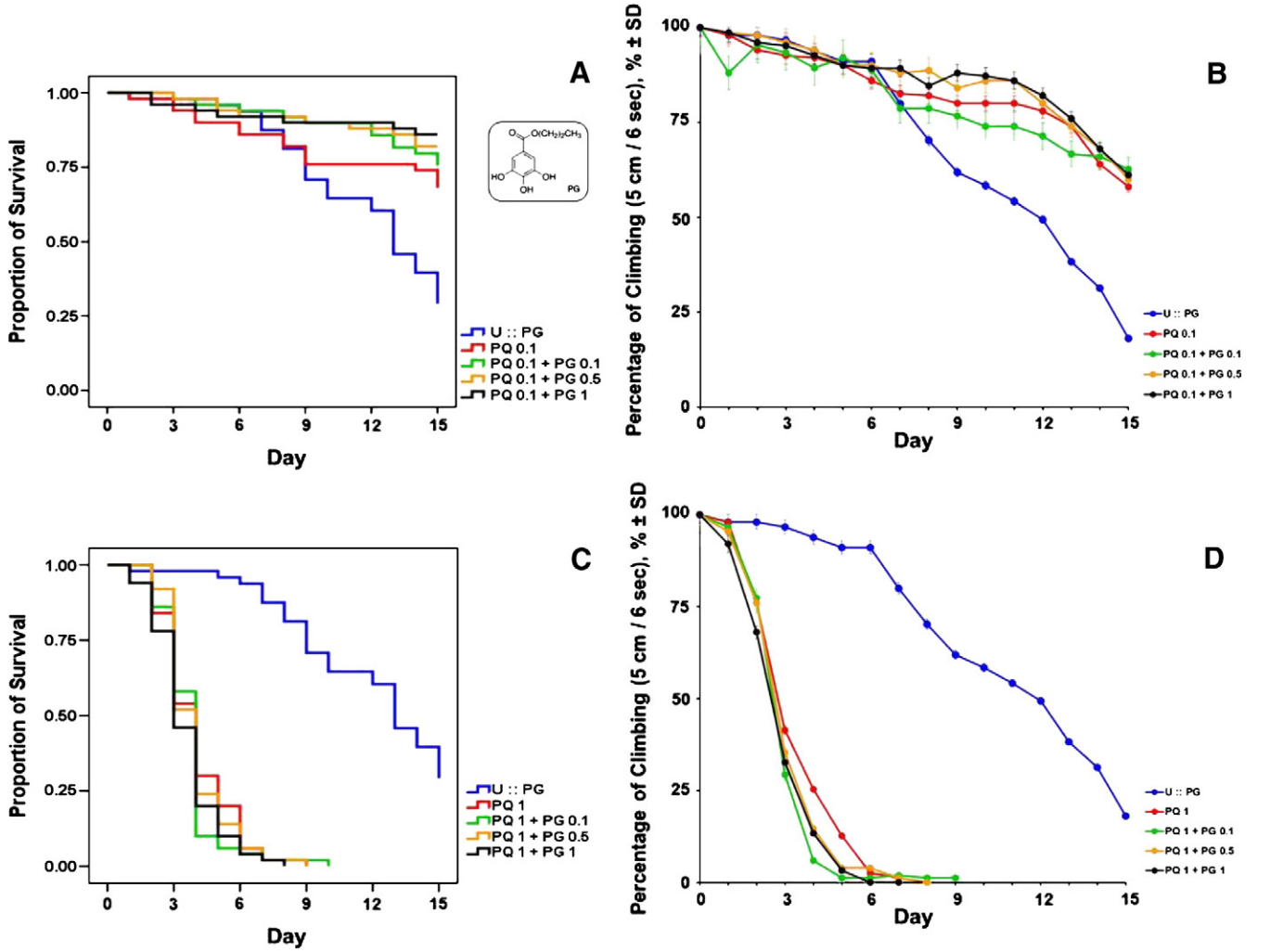

Fig. 3. Effect of PQ on the survival (A and C) and locomotor activity (B and D) of K-D *parkin Drosophila melanogaster* in the absence (0, blue bar) or presence of propyl gallate (PG:: 0.1 (green), 0.5 (orange bar), 1 (black). Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. The graphs (A and B) show significant differences between flies treated with (0.1 mM) PQ plus PG and PQ. These treatments also showed significant difference compared to untreated flies. No significant difference was observed between flies treated with (1 mM) PQ plus PG and PQ (C and D). Statistical comparisons between untreated and treated flies showed (A and C) $P<0.05$ by log-rank test and (B and D) $P<0.05$ by χ^2 test.

2.3. Antioxidant assay

The antioxidant assay was performed on virgin 2- to 3-day-old female flies collected overnight and kept on regular food medium. Subsequently, 50 females F1 were starved in empty vials for 3 h at 25 °C. Then, groups of five flies were placed in 10 vials containing a filter paper (Bio Rad Mini Trans-Blot 1703932) saturated with 1% GLU in dW for 24 h. Then, flies were fed with 200 μl fresh polyphenol solution (e.g., PG, GA, EC, EGCG) at (0.1–0.5 mM) alone or in combination with PQ for 15 days. Filters were changed daily. Red food dye (8 μl/1 ml) (Red food color McCormick) was added to ensure homogeneity and food intake. Survival proportion and locomotion assay (%) were rated at each interval of time.

2.4. Locomotion assay

The movement deficit assay was performed on both untreated and treated flies according to Ortega-Arellano et al. (2011). Briefly, (un) treated fF1 flies were placed in empty plastic vials. After a 10 min rest period, the flies were tapped to the bottom of the vials, and the number of flies able to climb 5 cm in 6 s was recorded at each interval of time. The assays were repeated three times at 1 min intervals. The scores are the mean of the numbers of flies at the top (n_{top}) and at the bottom (n_{bot}), expressed as percentages of the total number of flies (n_{tot}). Results are presented as the mean ± SD of the scores obtained in three independent assays. For each experiment, a climbing percent (%) was calculated, defined as $1/2[(n_{tot}+n_{top}-n_{bot})/n_{tot}]\times 100$, where n_{tot}, n_{top} and n_{bot} represent number of total flies, total number at the top and bottom, respectively. Data were shown as a mean ± standard deviation of the mean (SD). The chi square (χ^2) statistic was performed to compare proportion of percentage between independent groups. Differences were considered statistically significant at $P<0.05$.

2.5. Fluorescent microscopy

Dopaminergic neurons were visualized in transgenic flies using the GAL4/UAS system to express green fluorescent protein (GFP) and F1 K-D *parkin*, according to selection schedule (Fig. 1B, D and F). Adults expressing GFP and RNAi *parkin* under the control of TH-GAL4 (dopaminergic neuron expression) were fed with 0.1 or 1 mM PQ alone or combination with 0.1 mM PG up to reach 50% of survival. Whole mounts of dissected brains were examined for DAergic neuron morphology and number count per brain hemisphere was recorded using a fluorescent microscopy (Axiostar plus 50) in each treatment, according to Bonilla-Ramirez et al. (2011). The non-parametric Kruskal–Wallis test (i.e. equivalent to the one-way ANOVA) was performed to compare more than two independent groups. Differences were considered statistically significant at $P<0.001$. The Mann–Whitney *U* test was used to compare differences between two independent groups. Differences were considered statistically significant at $P<0.05$.

2.6. Survival test

Flies were treated chronically with PQ and polyphenols as described above for 15 days. Live flies were counted in groups of 5 flies per vial daily. Fifty flies per treatment were used. Survival curves were plotted using the Kaplan–Meier estimator. The statistical significance was calculated using the log-rank test within the portable IBM SPSS statistics 19 package program. The null hypothesis in all survival assays was that the presence of paraquat, polyphenols or genetic modifications in *Drosophila* made no difference to the survival of the flies in the absence of those reagents. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Low concentrations of paraquat alone increase life span and locomotor activity in knock-down parkin D. melanogaster against paraquat exposure

As a first approach, female ($n=50$) K-D *parkin D. melanogaster* flies were chronically exposed to increasing concentrations (0.1–20 mM) PQ. As shown in Fig. 2A and C, concentrations > 0.5 mM PQ were significantly toxic to the fly, but (0.1 and 0.25 mM) PQ increase survival

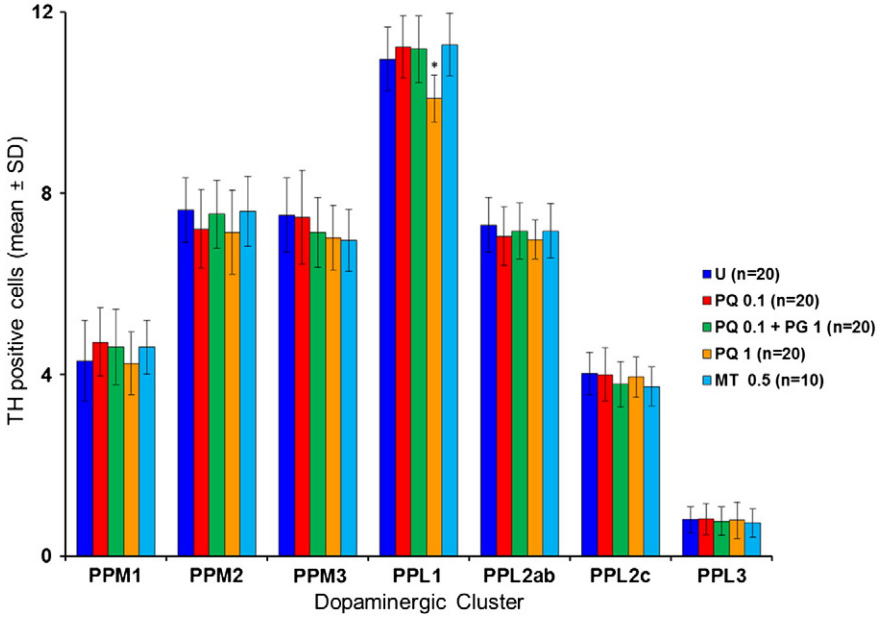

Fig. 4. High PQ concentration induces selective loss of protocerebral posterolateral (PPL1) subset of dopaminergic neurons. Untreated posterior brain fluorescent microscopy (A) of dopaminergic clusters in female K-D *parkin Drosophila melanogaster GFP$^+$TH-GAL4/UAS-RNAi parkin* adults. There were six clusters on the posterior side observed according to Mao and Davis (2009) and Bonilla-Ramirez et al. (2011): PPM1 (unpaired), PPM2 (paired), PPM3 (paired) (protocerebral posterior medial), PPL1, PPL2ab, PPL2c and PPL3 (paired) (protocerebral posterolateral). Average number of neurons of the medial and lateral dopaminergic neurons were scored for untreated and treated flies with (0.1, 1 mM) PQ, PQ plus (1 mM) PG and α-methyl-tyrosine (MT). ($n=$) represents the number of brain hemispheres examined (except PPM1) per treatment. *$P<0.05$, significant differences between treated and control neurons in each cluster. Error bars indicate ± SD.

compared to untreated K-D flies. Indeed, 50% flies exposed to 0.5, 1, 5, and 20 mM PQ perished at days 6, 4, 3, and 2 respectively. Interestingly, 50% untreated flies continued alive by day 13, whereas ~70% flies treated with 0.1 mM PQ and 50% flies treated with 0.25 mM PQ remained alive by day 15. The climbing performance inversely correlated with high PQ concentrations exposure and time but it directly correlated with low concentrations and time of PQ exposure (Fig. 2B and D).

3.2. Polyphenol propyl gallate (PG) increase survival and locomotor activity of knock-down parkin Drosophila melanogaster exposed to paraquat

Previous studies have shown that polyphenols such as PG, GA, EC and EGCG were effective antioxidants to prolong survival and restore locomotor activity in *D. melanogaster* wild type Canton-S against PQ (Jimenez-Del-Rio et al., 2010; Ortega-Arellano et al., 2011). We wanted therefore to test whether those polyphenols could potentiate the beneficial effect of low PQ concentrations in K-D *parkin* flies. As shown in Fig. 3, K-D flies treated with (0.1, 0.5, 1 mM) PG concentration in the presence of (0.1 mM) PQ increase significantly the survival (Fig. 3A) and locomotor activity (Fig. 3B) compared to untreated flies or treated with PQ or PG alone. In contrast, PG was not able to affect neither survival nor locomotor activity of K-D flies when co-treated with either (0.5 mM) or (1 mM) PQ (Fig. 3C and D). To further evaluate the effect of PQ and PG treatments on DAergic neurons in K-D flies, brains from treated flies TH-GAL4/UAS-RNAi-*parkin*/GFP flies (Fig. 1B) with either 0.1–1 mM PQ alone or in combination with 0.1 mM PG were examined under fluorescent microscopy at days 4–7 (Bonilla-Ramirez et al., 2011). As shown in Fig. 4, except flies treated with (1 mM) PQ, no significant differences in TH^+ neuronal cells were observed between untreated (U) and treated flies with (0.1 mM) PQ alone, (1 mM) PG alone, PQ plus PG, or MT, a TH inhibitor.

Because (0.1 mM) PQ did not affect the proportion of survival and percentage of climbing of K-D *parkin* flies by 7th day of exposure compared to untreated flies (Fig. 2A and B), we select this day as a time-frame to test whether (0.1 mM) PQ disposes K-D flies to increase survival and ameliorate climbing abilities when exposed to PQ and PG alone or in combination for additional 8 days. As shown in Fig. 5, flies pre-fed with (0.1 mM) PQ for 7 days and then fed with (0.25 mM) PQ for 8 days failed to increase survival (Fig. 5A) or altered locomotor capability (Fig. 5B) compared to flies exposed to (0.1–0.25 mM) PQ alone for 15 days. However, in flies pre-fed with (0.1 mM) PQ for the same initial period of time, i.e., 7 days, followed by a mixture of (0.25 mM) PQ and (1 mM) PG exposure for 8 days, a significant increase in survival rate was recorded when compared to flies treated with (0.25 mM) PQ alone under the same experimental condition (Fig. 5A). Climbing capabilities remained almost unaltered when compared between those treatments but were significantly different to untreated flies or treated with PG alone (Fig. 5B).

3.3. Polyphenols increase survival and locomotor activity of knock-down parkin Drosophila melanogaster exposed to low dose paraquat

Based on the above results, we were further interested to evaluate the effect of GA, EC, and EGCG on K-D *parkin Drosophila* flies treated with low concentration PQ. Unexpectedly, no significant difference in survival and climbing was observed between flies treated with (0.1, 0.5 mM) GA alone, GA plus PQ or untreated (Fig. 6A and B). In contrast, EC and EGCG showed a concentration dependent effect on survival (Fig. 7A and 8A), but whilst EC affected climbing in a concentration-independent fashion in flies treated in presence of PQ (Fig. 7B), EGCG significantly increase motor abilities (Fig. 8B) compared to flies treated with PQ or EGCG alone.

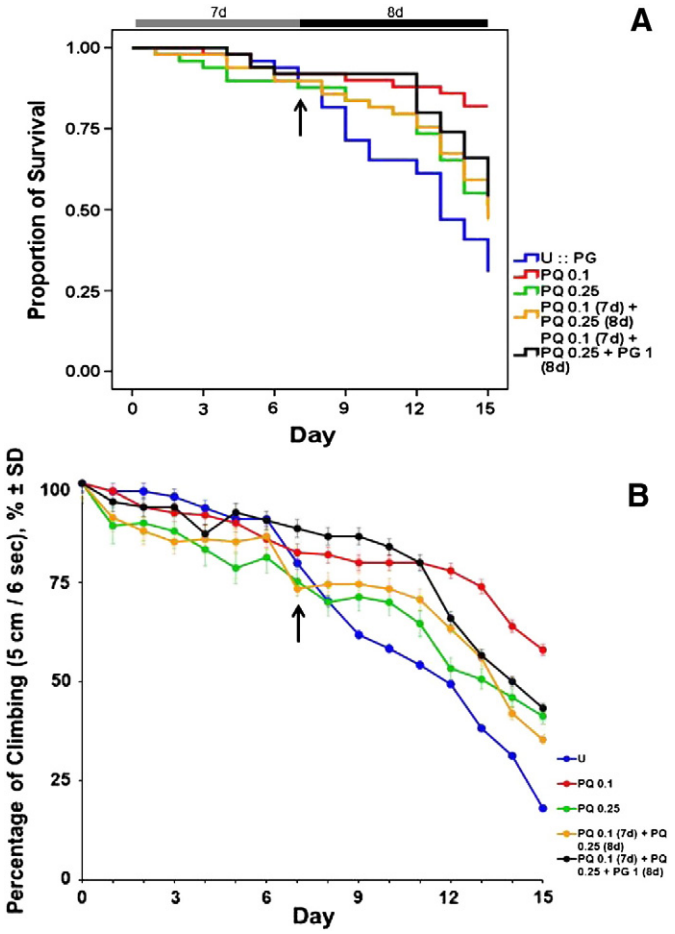

Fig. 5. Effect of pre-exposure dose of (0.1 mM) PQ for 7 days (arrow) and (0.25 mM) PQ alone (orange), or with (0.25 mM) PQ plus (1 mM) PG (black bar) for additional 8 days on the survival (A) and locomotor activity (B) of K-D *parkin Drosophila melanogaster*. Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A) $P<0.05$ by log-rank test and (B) $P<0.05$ by χ^2 test.

3.4. Low dose iron and chelator alone increase survival and locomotor activity of knock-down parkin Drosophila melanogaster

Previously, we reported that acute and chronic iron exposure in *Drosophila* reduced survival and locomotor activity (Bonilla-Ramirez et al., 2011; Jimenez-Del-Rio et al., 2010; Ortega-Arellano et al., 2011). Therefore, we were interested to establish whether chronically iron exposure affected K-D *parkin Drosophila* and whether chelating treatment might ameliorate fly survival and motor functionality. Effectively, K-D flies exposed to increasing iron concentrations (0.5–10 mM) dramatically decreased survival rate and locomotor activity compared to untreated flies (data not shown). Surprisingly, (0.1 mM) Fe and deferoxamine chelator (0.01 mM, DFO) alone significantly increase flies' proportion of survival (Fig. 9A) and locomotor performance (Fig. 9B) compared to untreated flies. However, chelator in the presence of iron reduced both survival and climbing to control values (Fig. 9A and B).

4. Discussion

This study report for the first time that polyphenols prolong life span ($P<0.05$ by log-rank test) and restore locomotor activity (i.e., climbing capability, $P<0.05$ by χ^2 test) in knock-down *parkin*, specifically in DAergic neurons in *Drosophila melanogaster* chronically exposed to PQ compared to untreated K-D *parkin* flies. In agreement with others (Greene et al., 2003; Pesah et al., 2004), we found that flies with

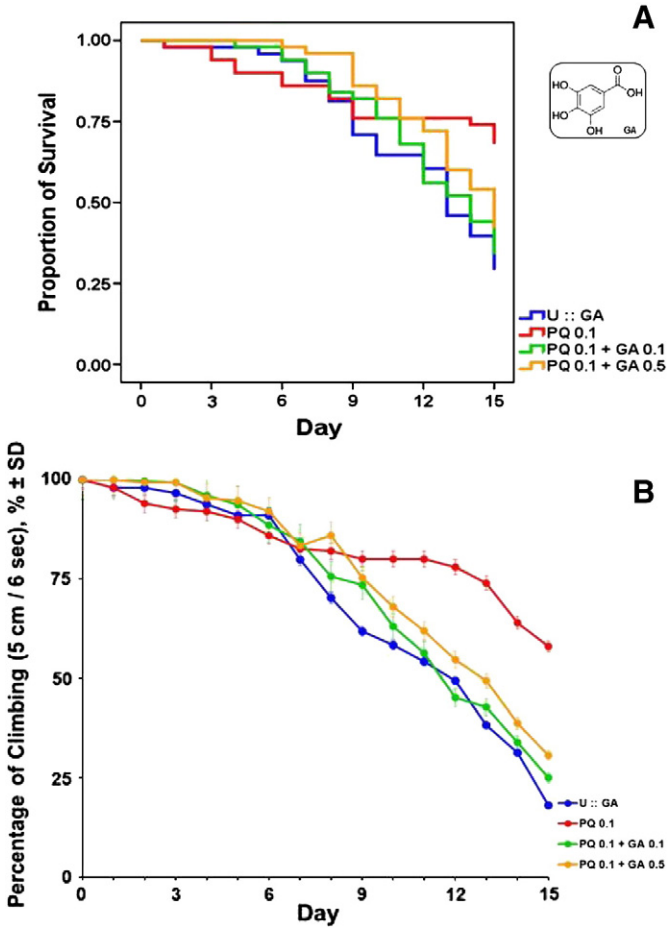

Fig. 6. Effect of PQ on the survival (A) and locomotor activity (B) of K-D *parkin Drosophila melanogaster* in the absence (0, blue bar) or presence of gallic acid (GA: 0.1 (green), 0.5 (orange bar)). Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A) $P<0.05$ by log-rank test and (B) $P<0.05$ by χ^2 test.

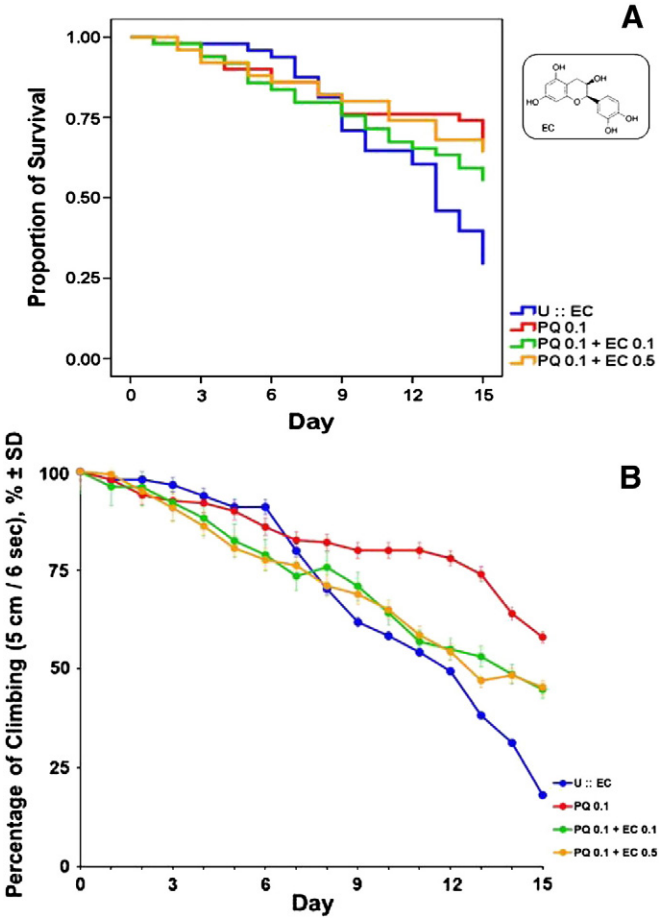

Fig. 7. Effect of PQ on the survival (A) and locomotor activity (B) of K-D *parkin Drosophila melanogaster* in the absence (0, blue bar) or presence of epicathecin (EC: 0.1 (green), 0.5 (orange bar)). Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A) $P<0.05$ by log-rank test and (B) $P<0.05$ by χ^2 test.

endogenous *parkin* gene inactivation by RNAi are sensitive to OS, present locomotor impairment and show no significant DAergic neuron loss. These observations suggest that Parkin might play an important role in protecting neuron cells from toxic stimuli (Jiang et al., 2004). Accordingly, it has been demonstrated that loss of Parkin function up-regulates the mediator of stress-induced cell death c-Jun N-terminal kinase (JNK), thereby contributing to the vulnerability of DAergic neurons to OS (Cha et al., 2005). Interestingly, loss of DJ-1 in *Drosophila* (Park et al., 2005), a stress sensor protein, showed severe defects in locomotor ability without loss of DA neurons. Taken together these data and ours suggest that endogenous Parkin and DJ-1 alterations are linked to neural dysfunction via mitochondria dysfunction (Burman et al., 2012; Thomas et al., 2011) rather than neural demise. This idea is further reinforced by the observation that either wild type (Bonilla-Ramirez et al., 2011) or RNAi *parkin* flies treated with MT induced locomotor impairment and reduced survival rate without significant dopaminergic neuronal loss. However, K-D *parkin* flies challenged with (>0.5 mM) PQ provoked a dramatic reduction in survival and climbing capabilities compared to untreated K-D flies. Together, these data comply with the notion that Parkin is essential for stress resistance and protection against neuronal injury and (probably) death. Surprisingly, we found that low PQ concentrations (e.g., 0.1 and 0.25 mM) prolong K-D *parkin* fly's life span and increase motor capabilities. One possible explanation is that low PQ induces an "adaptive stress response" in K-D *parkin* flies. This phenomenon is known as hormesis (Mattson, 2008). Although it is established that lifespan extension of *D. melanogaster* can be achieved through hormesis by repeated mild heat stress (Hercus et al., 2003; Sarup and Loeschcke, 2011), caloric restriction (Grandison et al., 2009) and/or reduced expression of proteins by RNAi (Liu et al., 2009), this is the first study to report PQ to positively affect lifespan and motor activity in knocked down *parkin* in *Drosophila* transgenic fly probably through hormesis. Accordingly, we found that PQ induced a biphasic dose–response in RNAi-*parkin* flies: a low dose stimulates survival/motor activities (i.e., beneficial effect) but high dose reduce lifespan/motor capabilities (i.e., inhibitory effect). These findings prompted us to test whether polyphenols such as PG might improve lifespan/motor activity of low PQ exposure on the transgenic fly, as previously reported for this polyphenol in wild type *Drosophila* Canton-S exposed to PQ (Jimenez-Del-Rio et al., 2010; Ortega-Arellano et al., 2011). Effectively, we found that PG in the presence of low PQ significantly increases the proportion of survival and thereby maintains locomotor activity comparable to flies treated with PQ alone, but a dramatic difference of these variables was observed compared to untreated flies. No positive effect on survival and climbing was observed when RNAi-flies were exposed to (1 mM) PQ and PG. Taken together these data suggest that low PQ (e.g., 0.1 mM) and PG synergistically increased lifespan and motor activities in K-D *parkin* flies. We then reasoned that since it is well known that exposure to low levels of one type of hermetic agent (e.g., PQ) can protect against more than one type of stress, we pre-exposed knocked down flies to (0.1 mM) PQ and then challenged them to mild concentration of

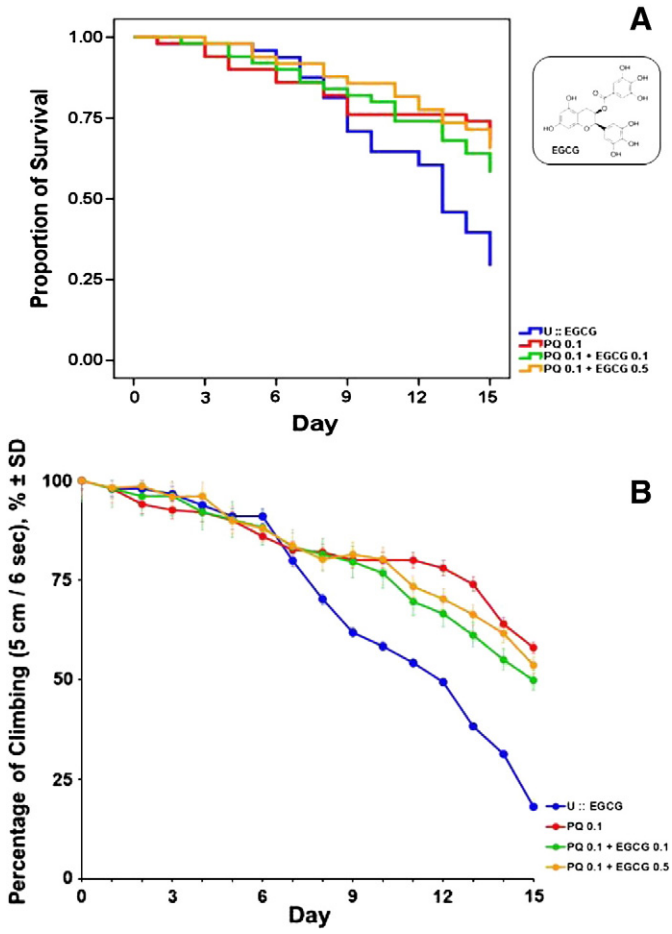

Fig. 8. Effect of PQ on the survival (A) and locomotor activity (B) of K-D *parkin Drosophila melanogaster* in the absence (0, blue bar) or presence of epigallocathecin gallate (EGCG: 0.1 (green), 0.5 (orange bar)). Female K-D flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A) $P<0.05$ by log-rank test and (B) $P<0.05$ by χ^2 test.

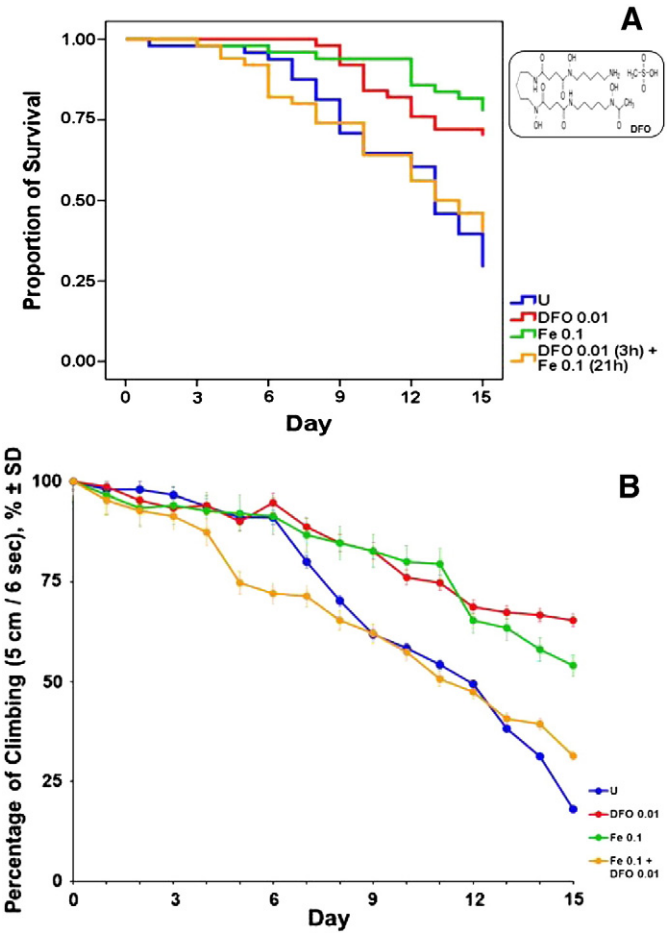

Fig. 9. Effect of iron ($FeSO_4$: 0.1 mM (green)) and deferoxamine (DFO: 0.01 mM (red)) alone or mixed (orange bar) on the survival (A) and locomotor activity (B) of K-D *parkin Drosophila melanogaster*. Female flies ($n = 50$ per treatment) were treated as described in Materials and methods. Statistical comparisons between untreated and treated flies showed (A) $P<0.05$ by log-rank test and (B) $P<0.05$ by χ^2 test.

PQ (e.g., 0.25 mM) for additional 8 days. We found that low PQ hermetic effect was diminished to comparable response of flies exposed to (0.25 mM) PQ alone but still significantly different to untreated flies. These data suggest that RNAi-*parkin* flies were not able to sustain mild PQ treatment. Since (1 mM) PG (8 days) modestly modify the fly's survival and climbing capabilities to (0.1 mM) PQ (7 days) plus (0.25) PQ (8 days) exposure, it is concluded that (i) K-D *parkin* flies are incapable to mount a proper hermetic response, (ii) *parkin* is essential for this adaptive stress response and (iii) co-administration of PG might positively, though modestly, affect lifespan and motor abilities of the transgenic flies. Similar to PG, EGCG positively affected survival and locomotor capabilities on RNAi-*parkin* flies. However, EC only ameliorates survival in the transgenic flies in the presence of PQ compared to untreated flies, but the synergistic effect between both polyphenols (GA, EC) and PQ on life span and climbing activity was nullify. Taken together these data suggest that differences in chemical structure and antioxidant activity based on the reduction potentials of polyphenols (McPhail et al., 2003; Villaño et al., 2007) might account for the observed synergistic effect with PQ. Our findings therefore suggest that exogenous antioxidant polyphenols can rescue pathology associated with compromised defenses to OS, but fail to extend the lifespan of RNAi-*parkin* flies. We therefore conclude that identification of specific polyphenols (e.g., PG, ECGC) that have synergistic effect with potentially pro-oxidant compounds (e.g., PQ) may lead to new treatment strategies for AR-JP/PD.

In conclusion, we have presented substantial evidence that suggests genetically altered *Drosophila melanogaster* as suitable model to study genetic and environmental (e.g., PQ, polyphenols) factors as causal and/or modulators in the development of PD. Most importantly, we have shown for the first time that low amounts of stressors, specifically PQ and iron, induce a health-promoting extending effect (e.g., life span, locomotor capabilities) in K-D *parkin* flies. Additionally, it is shown that selected polyphenols (e.g., PG, EGCG) are effective to protect K-D flies against PQ stress stimuli. Altogether our present results open new avenues for the screening, testing and development of novel antioxidant drugs against OS stimuli in neurodegenerative disorders.

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